Experimental control of pancreatic development and maintenance

Andrew M. Holland*†, Michael A. Hale*, Hideaki Kagami*‡, Robert E. Hammer§¶, and Raymond J. MacDonald*‖

Departments of *Molecular Biology and §Biochemistry, and the ¶Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75390

Edited by Eric N. Olson, University of Texas Southwestern Medical Center, Dallas, TX, and approved July 23, 2002 (received for review April 29, 2002)

To investigate the role of the HOX-like homeoprotein PDX1 in the formation and maintenance of the pancreas, we have genetically engineered mice so that the only source of PDX1 is a transgene that can be controlled by the application of tetracycline or its analogue doxycycline. In these mice the coding region for the tetracycline-regulated transactivator (tTA_{off}) has replaced the coding region of the endogenous *Pdx1* gene to ensure correct temporal and spatial expression of the regulatable transactivator. In the absence of doxycycline, tTA_{off} activates the transcription of a bicistronic transgene encoding PDX1 and an enhanced green fluorescent protein reporter, which acts as a visual marker of transgene expression in living cells. Expression of the transgene-encoded PDX1 rescues the *Pdx1*-null phenotype; the pancreata of these mice develop and function normally. The rescue is conditional; doxycycline-mediated repression of the transgenic *Pdx1* throughout gestation recapitulates the *Pdx1* null phenotype. Moreover, application of doxycycline at mid-pancreogenesis blocks further development. Adult animals of the rescue genotype that were treated with doxycycline for 3 weeks shut off *Pdx1* expression, decreased insulin production, and lost the ability to maintain glucose homeostasis. These results demonstrate the feasibility of controlling the formation of an organ during embryogenesis *in utero* and the maintenance of the mature organ through the experimental manipulation of a key developmental regulator.

The formation, growth, and maintenance of an organ are controlled by local and systemic morphogens, growth factors, and hormones. These endocrine factors work in turn by modulating common pathways that control organ-specific effectors, principally transcriptional regulators that activate sets of cell- and tissue-specific genes and define the phenotypes of differentiated cells. In many instances, key transcriptional regulators central to the genesis of an organ have been identified (e.g., reviews in refs. 1 and 2). *Pdx1* (*Ipf1, IDX1, STF1*), a gene encoding a HOX-like homeoprotein (3) and a member of the ParaHox gene cluster (4), plays such a role in pancreatic development.

Pancreogenesis of the mouse begins with the evagination of dorsal and ventral buds from the distal foregut endoderm about embryonic day 8.5–9 (5, 6). Growth and morphogenesis of the bud epithelium leads to a ramification of small ductules containing the precursor cells of the acini, ducts, and islets of Langerhans, the major differentiated tissues of the mature pancreas (7, 8). The islets form from dividing cells that escape the ductule epithelium, migrate through the surrounding mesenchyme, coalesce into endocrine cell masses, and differentiate into the four major islet cell types (8, 9). The majority of the dividing cells remain within the ductule epithelium and differentiate into either acinar cells, which synthesize digestive hydrolytic enzymes, or mature duct cells, which secrete the fluid that transports the digestive enzymes to the intestine. Pancreogenesis requires the integrated development of these endocrine and exocrine tissues.

Pdx1 is expressed throughout pancreatic development, from just before the onset of bud formation (3), through the periods of cell-type specification and differentiation (10), and persists in adult β cells (10–12) and at low levels in acinar cells (13). Inactivation of both *Pdx1* alleles blocks pancreatic development after the initial bud stage (14, 15). Thus, *Pdx1* is required for the elaboration of the emergent pancreatic buds and, therefore, for the formation of the islets, acini, and ducts. Ahlgren *et al.* (16) showed that Cre recombinase expressed from the insulin promoter gradually inactivated the *Pdx1* gene postnatally, and the mice became overtly diabetic at about 4 months of age. This article and more recent studies (12, 17, 18) demonstrated that *Pdx1* is also required for the maintenance of proper endocrine function of the mature pancreas. PDX1 binds and activates the promoters of the insulin (3) and elastase 1 (19) genes.

Because the germ-line disruption of the *Pdx1* gene blocks pancreogenesis at an early stage, it has not been possible to test whether PDX1 is required directly for the later stages of fetal pancreatic development. To begin investigating the role of PDX1 in the formation and maintenance of the pancreas, we have created mice in which all *Pdx1* expression can be suppressed at any time during the life cycle. In these mice the transcribed regions of both *Pdx1* alleles have been replaced by the coding sequence of the tetracycline-regulated transactivator (tTA_{off}) (20), which activates a PDX1-coding transgene driven by a heptameric tTA-binding site linked to a minimal promoter. Administration of the tetracycline analogue doxycycline inhibits the tTA_{off}-mediated activation of the transgene. PDX1 expressed from the transgene rescues the apancreatic phenotype of *Pdx1*-null fetuses, and doxycycline treatment during embryogenesis blocks the initiation or progression of pancreatic development, depending on the timing of doxycycline administration. Therefore, this experimental strategy can be used to block the developmental processes dependent on PDX1.

Materials and Methods

Knockin and Transgenic Mice. *$Pdx1^{tTA}$ knockin mice.* The vector for homologous recombination was constructed by using standard molecular biological techniques and contained the following functional regions (in order): the herpes simplex virus (HSV) *tk* gene; 4.5 kb of the 5′ flanking region of the *Pdx1* gene (from mouse strain SV129); the 51-bp 5′ untranslated region from the *Xenopus laevis* β-globin gene (21); the coding sequence of tTA_{off} from pUHD15–1 (20) for tetracycline regulation; the rabbit β-globin second intron and polyadenylation signal (20); the neomycin-resistance gene from pKO SelectNeo (Lexicon Genetics, The Woodlands, TX); and the 1.3-kb fragment from just downstream of the end of the second exon of *Pdx1*. Genomic clones bearing *Pdx1* sequences from mouse strain SV129 were a

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: tTA_{off}, tetracycline-regulated transactivator; EGFP, enhanced GFP; ES, embryonic stem; HSV, herpes simplex virus.

†Present address: Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia.

‡Present address: Oral and Maxillofacial Surgery, Nagoya University School of Medicine, Nagoya 466-8550, Japan.

‖To whom reprint requests should be addressed. E-mail: raymond.macdonald@utsouthwestern.edu.

www.pnas.org/cgi/doi/10.1073/pnas.192255099

gift from C. V. E. Wright, Vanderbilt University School of Medicine (15). All stem-cell manipulations were performed essentially as described (22, 23) by using R1 embryonic stem (ES) cells (24). Chimeric mice were generated from two independent ES cell clones with verified homologous recombination at the *Pdx1* locus.

Mice bearing a tTA-regulated Pdx1-EGFP transgene. The bicistronic transgene (Tg^{Pdx1}) contains the heptameric tetracycline operator and the cytomegalovirus minimal promoter with 83 bp of 5′ untranslated sequence from pUHD10–3 (20); a *Pdx1* minigene containing only 29 bp of the 5′ untranslated region and a shortened intron (now 500 bp); an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus (25) placed 83 nucleotides downstream of the *Pdx1* stop codon; the coding sequence for enhanced GFP (EGFP; CLONTECH); and a 225-bp fragment with the 3′ untranslated region and polyadenylation signal of the bovine growth hormone gene. The IRES was a gift from R. Behringer (University of Texas M. D. Anderson Cancer Center). Transgenic animals were generated by pronuclear injection (26, 27) and identified by PCR amplification of genomic DNA from tail biopsies and using primers to detect EGFP sequences (described below). The number of integrated transgene copies was measured by dot-blot hybridization with an EGFP hybridization probe.

Genotyping by PCR and Detecting mRNAs by RT-PCR. The various genotypes were all derived from crosses of mice that were both heterozygous for $Pdx1^{tTA}$ and hemizygous for Tg^{Pdx1}. The endogenous ($Pdx1^{+}$), the disrupted ($Pdx1^{tTA}$), and the transgenic (Tg^{Pdx1}) forms of *Pdx1* (as well as their mRNAs) were detected and distinguished by PCR amplification using gene- and allele-specific pairs of oligonucleotide primers. PCR amplification reactions included as template either genomic DNA isolated from tail biopsies for genotyping or cDNA synthesized by using reverse transcriptase and random hexanucleotide primers with total cellular RNA isolated from various organs for detecting mRNAs. Relative mRNA levels were calculated from PhosphorImager (Molecular Dynamics) quantification and adjusted by using the amount of PCR product for cytoplasmic actin mRNA. The conditions for PCR and the sequences of the oligonucleotide primers are available on request.

Total cellular RNA was purified from pancreatic tissue by using the guanidine thiocyanate procedure of Chirgwin *et al.* (28). RNA was isolated from all other mouse organs with Trizol (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Southern hybridization of PCR products and restriction-endonuclease-digested genomic DNA from ES cell clones was performed with standard protocols (e.g., ref. 29). The hybridization probes for Southern analyses of genomic DNA were prepared by PCR amplification of the appropriate genomic regions (see Fig. 1*A*).

Animal Care. Animals were housed under standard 12-h light/dark conditions and fed and watered *ad libitum*. Treatment with doxycycline was initiated by a single oral gavage (0.2 ml of 1 mg/ml doxycycline) and maintained with 1 mg/ml doxycycline in drinking water containing 5% sucrose. Gut organs were rapidly dissected *en bloc* from neonates and transferred to Carnoy's fixative for histological analysis. Fluorescence of EGFP was examined on unfixed visceral organs with a Leica MZFlIII stereo microscope (Leica, Deerfield, IL).

Immunocytochemistry and Immunofluorescence. Adult pancreata were fixed in Histochoice (Sigma) for processing, embedding in paraffin, and sectioning (5 μm). The following primary antibodies were used: guinea pig anti-human insulin at 1:1,000 (Dako), rabbit anti-human amylase at 1:1,000 (Sigma), rabbit anti-human glucagon at 1:200 (Dako), and rabbit anti-human somatostatin at 1:100 (Dako) in combination with either peroxidase-conjugated anti-rabbit or anti-guinea pig immunoglobulins for immunocytochemistry and with FITC-conjugated anti-guinea pig immunoglobulin (ICN) and Alexa fluor-568-conjugated goat anti-rabbit immunoglobulin (Molecular Probes) for immunofluorescence.

Results

To control expression of *Pdx1*, we engineered two genetic modifications (Fig. 1*A*): first, the introduction of a *Pdx1* transgene bearing a promoter that can be activated by the tetracycline-responsive tet-repressor/VP16 fusion transactivator (tTA_{off}) in the absence of tetracycline but not in its presence; second, the replacement of the coding region of the endogenous *Pdx1* locus with that of tTA_{off} by homologous recombination in ES cells. The substitution of tTA_{off} for *Pdx1* both inactivates the *Pdx1* allele and places tTA_{off} expression under the control of all of the endogenous *Pdx1* transcriptional regulatory sequences. A similar strategy has been used to manipulate the fetal development of melanoblasts and enteric neuroblasts from neural crest cells (30). In this report we show that this approach can be used to interrupt the formation or maintenance of an organ such as the pancreas.

The tTA Knockin Allele ($Pdx1^{tTA}$) Is Expressed in a Pancreas-Specific Manner. The knockin construct to replace the *Pdx1* coding region with tTA_{off} (Fig. 1*A*) contained 4.5 kb of the *Pdx1* 5′ flanking region including the transcriptional start site linked to the coding region of tTA_{off} (20) with an optimized 5′ untranslated region and translational start sequence (Fig. 1*A*). Homologous recombination in ES cells replaced the entire coding region (and intron) of *Pdx1*.

Two mouse lines containing the tTA-disrupted allele of *Pdx1* were generated from two independently derived ES cell clones (Fig. 1*B*). Properties of the mice derived from the two clones were indistinguishable and the lines derived from them were used interchangeably. Correct substitution at the *Pdx1* locus was verified by genomic Southern hybridization (Fig. 1*B* and data not shown). $Pdx1^{+/tTA}$ mice were identified by PCR analysis of DNA from tail biopsies by using primers that distinguish the wild-type and tTA-disrupted *Pdx1* alleles (Fig. 1*C*). The expression pattern of the $Pdx1^{tTA}$ locus, monitored by RT-PCR, was organ-specific and indistinguishable from that of unaltered *Pdx1* ($Pdx1^{+}$) (Fig. 1*D*). tTA mRNA was present in the pancreas of adult animals, but not in the other visceral organs or salivary glands tested.

$Pdx1^{tTA}$ Activates the *tetO-Pdx1* Transgene in Mice. The tTA-responsive *Pdx1* transgene was constructed from a tetracycline-regulated promoter (20) linked to a *Pdx1* minigene at an internal site of the first exon 29 nucleotides upstream of the *Pdx1* start codon (Fig. 1*A*). To monitor expression of the transgene, the coding sequence of EGFP was linked within the transcription unit through an internal ribosome entry site.

To determine whether the $Pdx1^{tTA}$ locus could activate the tTA-responsive *Pdx1* transgene (Tg^{Pdx1}) in mice, we mated $Pdx1^{+/tTA}$ mice of the 8-2 line (see Fig. 1*C*) with four independently derived Tg^{Pdx1} lines bearing single transgenic loci. The number of tandem transgene copies in these four lines varied from 10 to 22. Progeny from the crosses were genotyped by PCR (Fig. 2*A*) and examined to determine whether the transgenic loci were dependent on $Pdx1^{tTA}$ for pancreatic expression of the transgene in adult mice. The transgenes for two of the four lines had low basal levels of expression that increased in the presence of a $Pdx1^{tTA}$ allele by one (line 953) or two (line 956) orders of magnitude (Fig. 2*B*). Line 956, with the highest level of transgenic RNA, had about twice the level of Pdx1 RNA as animals with two normal *Pdx1* alleles. Expression of the transgene

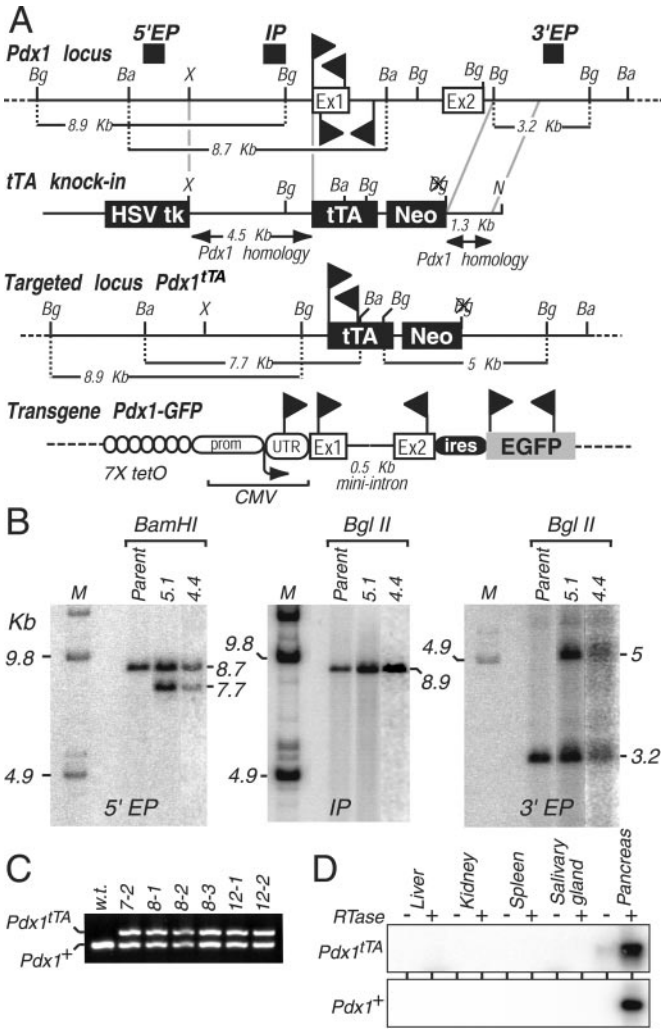

Fig. 1. Strategy for doxycycline-regulated expression of *Pdx1*. (*A*) The tTA knockin construct, the organization of the targeted *Pdx1* locus, and the *tetO-Pdx1/EGFP* transgene. The positions of the 5′ and 3′ external hybridization probes (*5′EP* and *3′EP*) and the internal probe (*IP*) are shown relative to restriction endonuclease sites and the two *Pdx1* exons. The tTA knockin construct contains the HSV thymidine kinase gene (*HSV tk*), 4.5-kb upstream and 1.3-kb downstream *Pdx1* homology regions, the tTA-coding region, and the neomycin-resistance gene (*Neo*). The lengths of the diagnostic restriction fragments [*Bgl*II (Bg), *Bam*HI (Ba), *Xba*I (X), and *Not*I (N)] are shown for the normal and the targeted *Pdx1* locus for the genomic Southern hybridizations of *B*. The tTA-responsive *Pdx1-EGFP* transgene contains seven repeats of the tTA-binding tet operator (*tetO*), the minimal cytomegalovirus (CMV) promoter with a short region of the cytomegalovirus 5′ untranslated region, a *Pdx1* minigene (shortened by deleting an internal region of the sole intron), the internal ribosome entry site (ires) derived from the encephalomyocarditis virus, the coding region of EGFP, and the 3′ untranslated region from the bovine growth hormone gene containing a poly(A) addition signal. The black flags show the positions of the PCR primers used to follow the transmission of the knock-in and transgene loci and to assay their expression by RT-PCR. (*B*) Homologous recombination of the Pdx1-tTA knockin construct in ES cells. The results of the Southern hybridization are shown for the R1 parent line and the two ES cell clones used to generate mice. The *Bam*HI fragment detected by the 5′EP is 8.7 kb for $Pdx1^{+}$ and 7.7 kb for $Pdx1^{tTA}$. The *Bgl*II fragment detected by the 3′EP is 3.2 kb for $Pdx1^{+}$ and 5 kb for $Pdx1^{tTA}$. The hybridization results from all three probes indicate a single insertion and only at the *Pdx1* locus. (*C*) PCR genotyping of nonchimeric mice for six lines generated from the two $Pdx1^{tTA}$ knockin ES cell clones distinguishes the $Pdx1^{+}$ and $Pdx1^{tTA}$ alleles. (*D*) tTA and Pdx1 RNAs were present selectively in the pancreas of adult $Pdx1^{+/tTA}$ mice of line 8-2 (*C*). Templates from cDNA synthesis reactions with and without reverse transcriptase (RTase) were used to show that amplified products were not caused by genomic DNA contamination of the RNAs. The PCR products for the tTA and Pdx1 RNAs were detected with gene-specific oligonucleotide hybridization probes.

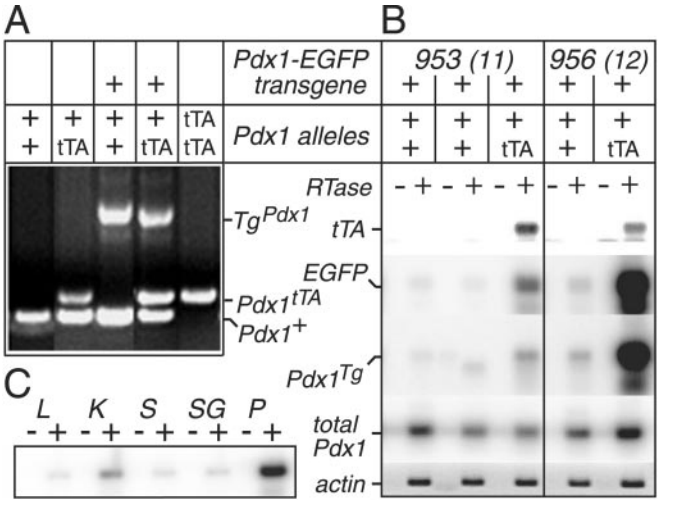

Fig. 2. Activation of the *tetO-Pdx1-EGFP* transgene (Tg^{Pdx1}) by the $Pdx1^{tTA}$ locus. (*A*) PCR genotyping identified mice with one wild-type and one tTA-disrupted allele of *Pdx1* and the presence of the transgene. (*B*) Pancreatic expression of Tg^{Pdx1} is dependent on tTA. Reverse-transcriptase-dependent detection of RNA transcripts for *tTA*, the *EGFP* region of the transgene, the *Pdx1* region of the transgene, all versions of *Pdx1* (endogenous locus and transgene), and cytoplasmic actin by PCR. (*C*) Detection of the transgenic mRNA for nonpancreatic organs was performed as in *B* with primers for the EGFP region of the transgenic mRNA. L, liver; K, kidney; S, spleen; SG, salivary gland; P, pancreas.

was detected at low levels in nonpancreatic tissues of adult mice (Fig. 2*C*).

The tet-Regulated Transgene Rescued the Homozygous *Pdx1*-Deficient Phenotype. To derive mice in which the sole source of PDX1 is regulatable by tetracycline, the *tetO-Pdx1* transgene must be able to rescue the *Pdx1*-deficient phenotype. Mice homozygous for the tTA-disrupted *Pdx1* locus ($Pdx1^{tTA/tTA}$) were born without a pancreas (Fig. 3 *C* and *D*). Only a small ductal remnant was present, located at the duodenal position from which the dorsal pancreatic bud normally derives. This apancreatic phenotype is the same as described in previous reports of *Pdx1* inactivation (14, 15, 31); thus, the expression of two tTA-expressing alleles does not alter the knockout phenotype. The presence of a transgenic locus in $Pdx1^{tTA/tTA}$ fetuses was sufficient to support the formation of a pancreas (Fig. 3 *E* and *F*). In this newborn animal the pancreas was approximately 50% the size of a normal neonatal pancreas. The gross morphology was otherwise normal, with gastric and splenic extensions comprising multiple, discrete lobules (Fig. 3*F*). The health and fertility of mice with the rescue genotype were indistinguishable from those of $Pdx1^{+/+}$ mice.

The presence of transgene-specific fluorescence of EGFP was conspicuous throughout the pancreatic tissue (Fig. 3*F′*) of animals with the rescue genotype ($Pdx1^{tTA/tTA}$;Tg^{Pdx1}), which is consistent with the persistence of PDX1 in acinar as well as endocrine cells of neonates (data not shown). Neither the pancreas from wild-type mice nor the small ductal remnant from $Pdx1^{tTA/tTA}$ mice fluoresced (Fig. 3 *B′* and *D′*), because of the absence of the Tg^{Pdx1} transgene. The pancreata of mice bearing only the transgene also did not fluoresce (not shown), verifying the lack of significant expression in the absence of tTA.

Normal Tissue Structure and Function of the Rescued Pancreas. The gross morphology of the adult pancreas from mice with the rescue genotype was normal (Fig. 4*A*). tTA-dependent expression of the Tg^{Pdx1} transgene in the adult pancreas could be detected by the widespread fluorescence of the EGFP reporter.

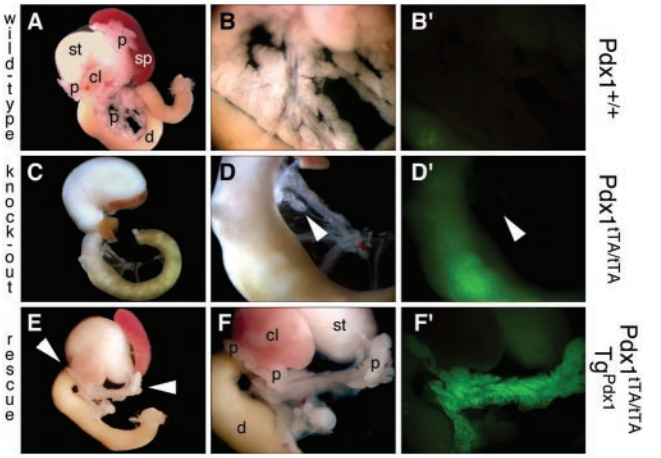

Fig. 3. The tTA-activated *Pdx1* transgene rescues the apancreatic phenotype of *Pdx1*-deficient mice. Low- (*A*, *C*, and *E*) and high- (*B*, *D*, and *F*) magnification views of viscera dissected from 2-day postpartum pups (P2) include stomach (st), spleen (sp), a caudal lobe of the liver (cl), duodenum (d), and pancreas (p). Wild type: The normal pancreas is organized as a collection of small lobules (*B*) spread throughout the duodenal loop with lobes that extend to the spleen and around the pyloric region of the stomach (*A*). Pancreatic tissue from mice lacking the transgene does not fluoresce (*B′*). Knockout: Viscera from newborn $Pdx1^{tTA/tTA}$ mice lack pancreatic tissue, but otherwise seem normal (*C*). A very small epithelial remnant is present at the normal attachment site of the dorsal lobe to the duodenum (arrowhead in *D*). No EGFP fluorescence is detectable in the remnant (arrowhead in *D′*). Endogenous fluorescence from the duodenum (*D′*) is independent of the Tg^{Pdx1} transgene, has a different hue than EGFP, and arises from the luminal content of partially digested milk, which varies among neonates. Rescue: The pancreas of the rescue genotype is present in the duodenal loop and has splenic and gastric lobes (arrowheads in *E* and p in *F*). The pancreas has EGFP fluorescence (*F′*).

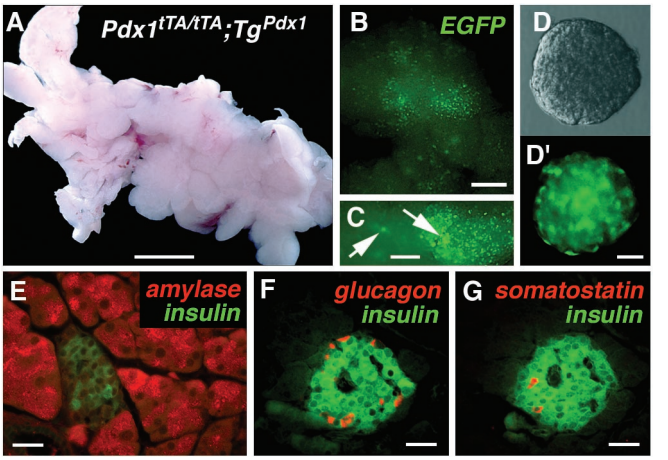

Fig. 4. The tetracycline-regulated *Pdx1* transgene supports the formation of a normal adult pancreas. (*A*) Normal gross morphology of the pancreas from a 9-week-old adult mouse with the rescue genotype ($Pdx1^{tTA/tTA};Tg^{Pdx1}$). (Bar = 1 mm.) (*B* and *C*) Two magnified views showing widespread EGFP fluorescence directed by the *tetO-Pdx1/EGFP* transgene. (Bars: 200 μm in *B*, 50 μm in *C*.) The arrows in *C* indicate clusters of fluorescing cells that may be islets of Langerhans. (*D* and *D′*) Bright-field (*D*) and fluorescence (*D′*) views show EGFP fluorescence in most cells of an islet isolated from a rescued adult pancreas. (Bar = 25 μm.) Islets were isolated as described (39) and cultured for 48 h in high-glucose medium to reduce autofluorescence. (*E*) Insulin is present in the islet and amylase in acini with normal morphology. The punctate amylase-immunofluorescence detects zymogen granules segregated near the apical lumens of acinar cells. Consecutive sections through an islet from a rescue mouse show the presence of an insulin-staining core and more peripheral glucagon (*F*) and somatostatin (*G*) cells. (Bars = 25 μm.)

Regions in the plane of focus at high magnification showed the fluorescence of individual cells (Fig. 4 *B* and *C*) and occasional cell clusters (Fig. 4*C*), presumably islets. Isolated islets fluoresced intensely (Fig. 4 *D* and *D′*), consistent with the relatively high level of endogenous PDX1 in mature β cells. Exocrine and endocrine tissue architecture was normal (Fig. 4*E*). Acini were well formed, with polarized acinar cells containing high levels of amylase localized to zymogen granules clustered in the apical regions of cells organized around a central lumen. Insulin immunofluorescence was extensive throughout the central core of the islets (Fig. 4 *F* and *G*). Glucagon- (Fig. 4*F*) and somatostatin- (Fig. 4*G*) containing cells were fewer and more peripheral, which are indicative of normal islet architecture. Moreover, pancreatic endocrine function was effective, because rescue mice had blood glucose levels within normal ranges (Fig. 6*D* and data not shown) and respond appropriately to a glucose challenge (see Fig. 6*D*).

Embryonic Pancreatic Development Can Be Blocked by Doxycycline. Treatment of pregnant mice with doxycycline from the first day of pregnancy prevented the formation of a pancreas in fetuses of the rescue genotype (Fig. 5*B*). The small pancreatic remnants were similar to those of $Pdx1^{-/-}$ newborns (compare Fig. 5 *A′* and *B′*). In contrast, neonates with at least one normal *Pdx1* allele generally had normal amounts of pancreatic tissue (Fig. 5*C*). We examined a total of five newborn rescue pups from four independent mothers treated with doxycycline to ensure that the apancreatic phenotype was caused by doxycycline-mediated repression of the transgene. None had pancreatic tissue.

Between the appearance of the pancreatic bud at embryonic days 9 and 13, normal pancreatic epithelium grows and forms a complex of ductules, but the differentiation of acinar and islet cells is not yet begun (6, 7). Treatment of mothers beginning on embryonic day 11.5 of gestation through parturition arrested the pancreatic development of fetuses with the rescue genotype about 36 h later. At birth the undeveloped pancreatic remnant consisted of a large and extended duct (Fig. 5*D*) with several terminal, aborted, ductal buds (Fig. 5*E*). A single layer of primitive epithelial cells lined the duct and neither acinar nor islet tissue was detected (Fig. 5*F*). Therefore, pancreatic development *in utero* can be either prevented or interrupted, depending on the timing of doxycycline-mediated suppression of *Pdx1*.

Doxycycline Treatment of Adult Mice Induces Glucose Intolerance. To determine whether expression of the tet-regulated *Pdx1* transgene could be inhibited by doxycycline in adult rescue mice, we measured the levels of transgenic mRNA in the pancreas of mice before and after 6 days of treatment with doxycycline (Fig. 6*A*). Doxycycline decreased the level of the transgenic Pdx1-EGFP mRNA an average of 16-fold (SEM = ±4.6-fold) for three animals containing one $Pdx1^{tTA}$ allele and the Tg^{Pdx1} transgene. Treatment for an additional 24 days caused a further 2- to 3-fold decrease (data not shown).

To examine the long-term effects of PDX1 depletion on endocrine and exocrine functions, rescue mice were treated with doxycycline for 21 days. After treatment, insulin mRNA decreased 10-fold, whereas glucagon mRNA remained constant (Fig. 6*C*). PDX1 was undetectable by immunostaining and insulin was greatly depleted (Fig. 6*B*), and β-cell control of glucose homeostasis was severely impaired (Fig. 6*D*). Fasting blood glucose levels were increased 4-fold to diabetic levels (Fig. 6*D*). $Pdx1^{+/+}$ mice (with or without a *Pdx1* transgene) had a normal response to a glucose challenge, and heterozygous *Pdx1* mice had a partially impaired glucose tolerance (Fig. 6*D*), as described (17), and these responses were not affected by doxycycline treatment (data not shown). Whereas the response to a glucose challenge in untreated rescue mice was normal, the

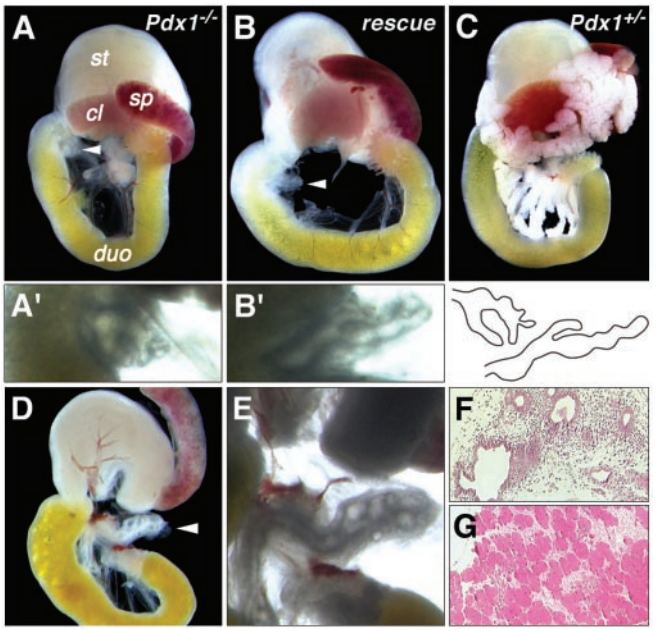

Fig. 5. Treatment of pregnant mice with doxycycline blocks pancreogenesis. (*A*) Viscera from a *Pdx1*-null (*Pdx1*$^{tTA/tTA}$) neonate (st, stomach; sp, spleen; cl, a caudal lobe of the liver; duo, duodenum). (*B*) Viscera from a neonate of the rescue genotype after doxycycline treatment of the mother from the day of conception. The arrowheads of *A* and *B* indicate the pancreatic remnants. (*C*) Viscera from a *Pdx1*$^{+/tTA}$ neonate with a normal pancreas. The animals of *A*–*C* were littermates. (*A*′ and *B*′) Enlarged bright-field views of the pancreatic remnants of *A* and *B*, respectively. Tracings of the epithelial caeca of the pancreatic remnants in *A*′ and *B*′ are shown in the image at the *Middle Right*. (*D* and *E*) An extended pancreatic ductal remnant (arrowhead) of a neonate treated with doxycycline from embryonic day 11.5. Aborted ductal budding at the distal end of the duct structure can be seen in *E*. (*F* and *G*) Histology of the ductal remnant (*F*) stained with hematoxylin and eosin compared with that of a normal neonatal pancreas (*G*). Crude ductal structures in the remnant replace the plump eosinophilic acini of the normal pancreas.

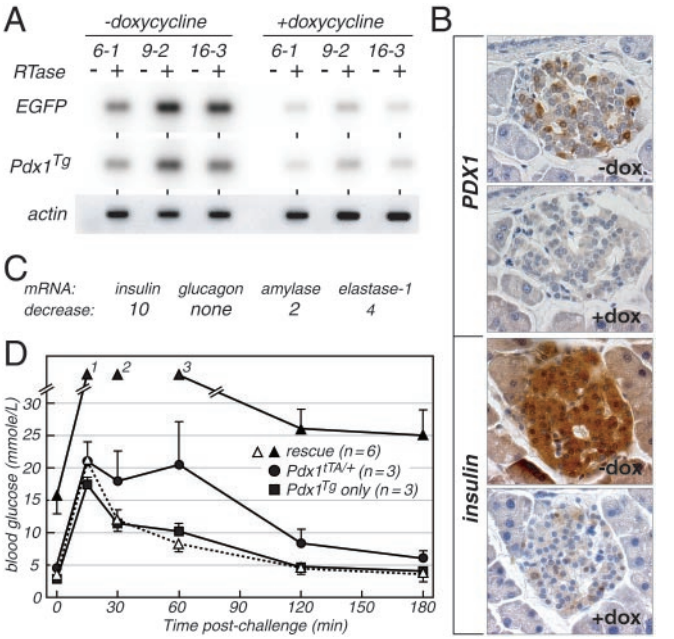

Fig. 6. PDX1 deficiency caused by treating mice of the rescue genotype with doxycycline creates a diabetic phenotype. (*A*) Doxycycline decreased the level of transgenic mRNA in adult *Pdx1*$^{tTA/tTA}$;*Tg*^{Pdx1} mice. Before doxycycline treatment, pancreatic tissue samples were obtained by laparotomy from three adult males. After 7 days of recovery, doxycycline was administered for 6 days. RNA was isolated from the pancreatic tissue obtained before and after doxycycline treatment. The level of transgenic mRNA was measured by RT-PCR for both the *Pdx1* and EGFP regions of the bicistronic mRNA. (*B*) Doxycycline causes the loss of β-cell PDX1 and insulin after 21 days. In the absence of doxycycline treatment, the normal high level of insulin causes bleeding of the immunostain into adjacent acinar tissue. (*C*) Treatment with doxycycline for 21 days lowers the levels of mRNAs for exocrine enzymes as well as for islet hormones. mRNA levels were quantified as for *A*. (*D*) Glucose handling in doxycycline-treated adults is greatly compromised. All values are for adults treated for 14 days with doxycycline, except for the values designated with the open triangles and dashed lines, which are for the rescue animals before doxycycline treatment. The values marked with numbers exceeded the uppermost limit of the glucometer (33.3 mM) for four (1), one (2), and one (3) of the six animals, so that average values could not be calculated.

response by doxycycline-treated rescue mice was defective (Fig. 6*D*).

Doxycycline-mediated repression of *Pdx1* also led to a modest decrease in the expression of two genes encoding acinar-specific secretory enzymes. Amylase and elastase 1 mRNA levels were decreased 2- and 4-fold, respectively, by a 21-day treatment with doxycycline (Fig. 6*C*).

Discussion

The results from this study show that control of a key developmental regulator through an optimized tetracycline-regulation system can be used to manipulate the formation of a compound gland during fetal organogenesis and the maintenance of glandular function in mature animals. We incorporated several modifications to help ensure that regulated expression of PDX1 from a transgene could compensate for the absence of both endogenous *Pdx1* alleles. The correct timing of activation of the *Pdx1* transgene was optimized by inserting the coding region of the tTA transcriptional activator into the *Pdx1* locus to place tTA under control of the endogenous *Pdx1* promoter. Sufficient tTA was produced to activate the *Pdx1* transgene to an effective level. By screening multiple lines bearing the tetracycline-responsive *Pdx1* transgene, we were able to identify several that could restore fetal pancreatic development in the absence of a functional endogenous *Pdx1* allele.

The rescue of pancreatic development by the *Pdx1* transgene is conditional; administration of doxycycline to pregnant mice before the time of fetal *Pdx1* gene activation prevents the formation of both the dorsal and ventral pancreatic rudiments in fetuses with the rescue genotype by blocking the production of PDX1. These and previous results (14, 15) show that PDX1 is required for the growth of the nascent pancreatic epithelium and its morphogenesis into undifferentiated ductules. We asked whether *Pdx1* is also required for the next developmental transition, the differentiation of endocrine and exocrine cells from ductule precursors. Indeed, inhibition of *Pdx1* expression before cell differentiation, which begins at embryonic day 13, blocked the formation of acini and islets. Therefore, PDX1 is also necessary for the onset of cellular differentiation, possibly for the transcriptional activation of the battery of transcription factor genes (e.g., ref. 32) that direct the formation of individual exocrine and endocrine cell lineages.

In adult mice, depletion of PDX1 by doxycycline treatment for 3 weeks disturbed the function of both endocrine and exocrine tissues. The level of insulin protein and mRNA decreased manyfold. Not all of the insulin-expressing islet cells behaved uniformly, however; a small fraction retained strong immunostaining for insulin, similar to normal β cells. These cells may represent the descendants of the *Pdx1*-independent, insulin-expressing cells (33) that appear in the early pancreatic buds of normal and *Pdx1*-deficient embryos (14).

Adult mice with the rescue genotype maintained both nonfasting and fasting blood glucose levels within normal ranges and were able to respond normally to a glucose challenge. Doxycycline-mediated repression of *Pdx1* in these mice led to an increase of fasting blood glucose to diabetic levels and a severely impaired ability to respond to a glucose challenge. Although earlier *Pdx1* knock-down studies in the mature pancreas indicated a role for PDX1 in the maintenance of pancreatic function, these studies were hampered by their dependence on activation of the insulin promoter to repress *Pdx1* (12, 18). The striking physiological effect observed in the present study demonstrates that this is a unique model system to study the role of *Pdx1* in adult β-cell function.

The decrease of amylase and elastase mRNA levels by doxycycline treatment of adult rescue mice suggests that PDX1 participates in the maintenance of acinar function as well. The effect on the two representative acinar enzyme mRNAs was modest (2- to 3-fold), however, and the requirement in adult acinar tissue may not be a cell-autonomous one. Although PDX1 is required for the formation of acinar cells (15, 31) and persists at high levels in acinar nuclei perinatally (H.K. and R.J.M., unpublished data), it is present only at low levels in adult acinar cell nuclei (13) and its overexpression disrupts acinar function (34). Consequently, the continued presence of PDX1 in acinar cells may not be necessary for their function in the mature gland. Because efficient amylase gene transcription is dependent on insulin (35, 36), the decrease of amylase mRNA accumulation may be an indirect effect mediated through decreased insulin production. Although the level of elastase enzyme is affected by long-term diabetes (37), elastase I gene transcription is not affected in the short term by insulin (38). A direct effect on the elastase I gene would be consistent with the demonstrated ability of PDX1 to bind and activate the transcriptional enhancer of the elastase I gene in acinar cells (19). Thus, PDX1 may play both direct and indirect roles in maintaining acinar function. Proof for a direct, cell-autonomous function awaits the acinar-specific inactivation of *Pdx1*.

We anticipate that the ability to block *Pdx1* gene expression by doxycycline will be useful to define temporally distinct roles for PDX1 during fetal and postnatal pancreatic development, during the maintenance of the mature organ, and during times of stress such as endocrine and exocrine tissue destruction and regeneration. The $Pdx1^{tTA}$ locus should also be useful to control the expression of other tTA-responsive transgenes selectively in *Pdx1*-expressing cells.

We thank Leonard C. Harrison, in whose laboratory A.M.H. completed some of this work, for support and helpful discussions. We also thank Richard Behringer for the encephalomyocarditis virus internal ribosome entry site, Chris V. E. Wright for genomic clones of mouse *Pdx1*, Mark Henkemeyer for R1 embryonic stem cells, M. Gossen, L. Henighausen, and H. Bujard for the tetracycline regulation constructs, and Galvin Swift for helpful criticisms of the manuscript. This work was initiated by a grant from the Texas Higher Education Coordinating Board and was supported by a Juvenile Diabetes International fellowship (to A.M.H.), an American Diabetes Association Mentor-based Postdoctoral Fellowship, and Research Grant DK-55266 from the National Institutes of Health.

1. Srivastava, D. & Olson, E. N. (2000) *Nature (London)* **407,** 221–226.
2. Scully, K. & Rosenfeld, M. G. (2002) *Science* **295,** 2231–2235.
3. Ohlsson, H., Karlsson, K. & Edlund, T. (1993) *EMBO J.* **12,** 4251–4259.
4. Pollard, S. L. & Holland, P. W. H. (2000) *Curr. Biol.* **10,** 1059–1062.
5. Wessells, N. K. & Cohen, J. H. (1967) *Dev. Biol.* **15,** 237–270.
6. Slack, J. M. W. (1995) *Development (Cambridge, U.K.)* **121,** 1569–1580.
7. Pictet, R. L., Clark, W. R., Williams, R. H. & Rutter, W. J. (1972) *Dev. Biol.* **29,** 436–467.
8. Pictet, R. & Rutter, W. J. (1972) in *Handbook of Physiology*, eds. Steiner, D. F. & Freinkel, N. (Williams & Wilkins, Baltimore), Sect. 7, Vol. 1, pp. 25–66.
9. Deltour, L., Leduque, P., Paldi, A., Ripoche, M. A., Dubois, P. & Jami, J. (1991) *Development (Cambridge, U.K.)* **112,** 1115–1121.
10. Guz, Y., Montminy, M. R., Stein, R., Leonard, J., Gamer, L. W., Wright, C. V. E. & Teitelman, G. (1995) *Development (Cambridge, U.K.)* **121,** 11–18.
11. Miller, C. P., McGehee, R. E. & Habener, J. F. (1994) *EMBO J.* **13,** 1145–1156.
12. Lottmann, H., Vanselow, J., Hessabi, B. & Walther, R. (2001) *J. Mol. Med.* **79,** 321–328.
13. Wu, K.-L., Gannon, M., Peshavaria, M., Offield, M. F., Henderson, E., Ray, M., Marks, A., Gamer, L. W., Wright, C. V. E. & Stein, R. (1997) *Mol. Cell. Biol.* **17,** 6002–6013.
14. Ahlgren, U., Jonsson, J. & Edlund, H. (1996) *Development (Cambridge, U.K.)* **122,** 1409–1416.
15. Offield, M. F., Jetton, J. L., Labosky, P. A., Ray, M., Stein, R. W., Magnuson, M. A., Hogan, B. L. M. & Wright, C. V. E. (1996) *Development (Cambridge, U.K.)* **122,** 983–995.
16. Ahlgren, U., Jonsson, J., Jonsson, L., Simu, K. & Edlund, H. (1998) *Genes Dev.* **12,** 1763–1768.
17. Dutta, S., Bonner-Weir, S., Montminy, M. & Wright, C. (1998) *Nature (London)* **392,** 560 (lett.).
18. Thomas, M. K., Devon, O. N., Lee, J. H., Peter, A., Schlosser, D. A., Tenser, M. S. & Habener, J. F. (2001) *J. Clin. Invest.* **108,** 319–329.
19. Swift, G. H., Liu, Y., Rose, S. D., Bischof, L., Steelman, S., Buchberg, A. M., Wright, C. V. E. & MacDonald, R. J. (1998) *Mol. Cell. Biol.* **18,** 5109–5120.
20. Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
21. Falcone, D. & Andrews, D. W. (1991) *Mol. Cell. Biol.* **11,** 2656–2664.
22. Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E. & Herz, J. (1993) *J. Clin. Invest.* **92,** 883–893.
23. Yanagisawa, H., Yanagisawa, M., Kapur, R. P., Richardson, J. A., Williams, S. C., Clouthier, D. E., de Wit, D., Emoto, N. & Hammer, R. E. (1998) *Development (Cambridge, U.K.)* **125,** 825–836.
24. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. & Roder, J. C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8424–8428.
25. Kim, D. G., Kang, H. M., Jang, S. K. & Shin, H. S. (1992) *Mol. Cell. Biol.* **12,** 3636–3643.
26. Brinster, R. L., Chen, H. Y., Trumbauer, M. E., Yagle, M. K. & Palmiter, R. D. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4438–4442.
27. Swift, G. H., Hammer, R. E., MacDonald, R. J. & Brinster, R. L. (1984) *Cell* **38,** 639–646.
28. MacDonald, R. J., Swift, G. H., Przybyla, A. E. & Chirgwin, J. M. (1987) *Methods Enzymol.* **152,** 219–227.
29. Hale, M. A. & Garrard, W. T. (1998) *Mol. Immunol.* **35,** 609–620.
30. Shin, M. K., Levorse, J. M., Ingram, R. S. & Tilghman, S. M. (1999) *Nature (London)* **402,** 496–501.
31. Jonsson, J., Carlsson, L., Edlund, T. & Edlund, H. (1994) *Nature (London)* **371,** 606–609.
32. Schwitzgebel, V. M. (2001) *Mol. Cell. Endocrinol.* **185,** 99–108.
33. Larsson, L. I. (1998) *Microsc. Res. Tech.* **43,** 284–291.
34. Heller, R. S., Stoffers, D. A., Bock, T., Svenstrup, K., Jensen, J., Horn, T., Miller, C. P., Habener, J. F., Madsen, O. D. & Serup, P. (2001) *Diabetes* **50,** 1553–1561.
35. Osborn, L., Rosenberg, M. P., Keller, S. A. & Meisler, M. H. (1987) *Mol. Cell. Biol.* **7,** 326–334.
36. Korc, M., Owerbach, D., Quinto, C. & Rutter, W. J. (1981) *Science* **213,** 351–353.
37. Hardt, P. D., Krauss, A., Bretz, L., Porsch-Ozcurumez, M., Schnell-Kretschmer, H., Maser, E., Bretzel, R. G., Zekhorn, T. & Klor, H. U. (2000) *Acta Diabetol.* **37,** 105–110.
38. Keller, S. A., Rosenberg, M. P., Johnson, T. M., Howard, G. & Meisler, M. H. (1990) *Genes Dev.* **4,** 1316–1321.
39. Lake, S. P., Anderson, J., Chamberlain, J., Gardner, S. J., Bell, P. R. & James, R. F. (1987) *Transplantation* **43,** 805–808.